0273-2289/04/113/0261–0272/$25.00

Immobilized Enzyme Studies in a Microscale Bioreactor

Francis Jones,[1] Scott Forrest,[2] Jim Palmer,[2] Zonghuan Lu,[2] John Elmore,[2] and Bill B. Elmore*,[2]

[1]Chemical and Environmental Engineering, The University of Tennessee at Chattanooga, 615 MacCallie Avenue, Chattanooga, TN 37403-2598; and [2]Department of Chemical Engineering, Louisiana Tech University, 600 W. Arizona Avenue, Ruston, LA 71272, E-mail: belmore@coes.latech.edu

Abstract

Novel microreactors with immobilized enzymes were fabricated using both silicon and polymer-based microfabrication techniques. The effectiveness of these reactors was examined along with their behavior over time. Urease enzyme was successfully incorporated into microchannels of a polymeric matrix of polydimethylsiloxane and through layer-bylayer self-assembly techniques onto silicon. The fabricated microchannels had cross-sectional dimensions ranging from tens to hundreds of micrometers in width and height. The experimental results for continuous-flow microreactors are reported for the conversion of urea to ammonia by urease enzyme. Urea conversions of >90% were observed.

Index Entries: Microscale bioreactor; polydimethylsiloxane microreactor; immobilized enzymes; urease enzyme; silicon wafer.

Introduction

The field of chemical process miniaturization is growing at a rapid pace with promising improvements in process control, product quality, and safety, *(1,2)*. Microreactors typically have fluidic conduits or channels on the order of tens to hundreds of micrometers. With large surface area–to–volume ratios, rapid heat and mass transfer can be accomplished with accompanying improvements in yield and selectivity in reactive systems. Microscale devices are also being examined as a platform for traditional unit operations such as membrane reactors in which a rapid removal of reaction-inhibiting products can significantly boost product yields *(3–6)*.

*Author to whom all correspondence and reprint requests should be addressed.

While microscale devices and systems are typically fabricated from silicon, alternative materials are being examined as suitable supports. Microsystem features as small as 1 μm may be fabricated precisely by using a variety of etching, molding, and milling techniques. Since enzymatic reactions typically occur at moderate operating conditions (moderate pressures and ambient temperatures), plastics may be considered an inexpensive alternative to silicon for use as microreactor fabrication material.

In this article, we report on the fabrication and performance of microreactors constructed of silicon and polydimethylsiloxane (PDMS). The resulting structures contain immobilized enzymes for converting biochemical substrates to useful products or for breaking down organics into waste streams.

Materials and Methods

Materials Used

Urease (EC 3.5.1.5 Type IX, Sigma-Aldrich: from Jack Beans) was used throughout the experiments. Before immobilizing urease onto the microreactor systems, the enzyme was evaluated for activity in the chosen buffer system (Tris[hydroxymethyl]aminomethane [THAM]). Free enzyme tests of the urease showed an approximate activity of 44,800 U/g of solid.

Batch studies for evaluating immobilized enzyme activity and properties of the "bioplastic" (urease entrapped in PDMS) material were conducted in 250-mL shake flasks in an environmentally controlled shaker/incubator.

Continuous studies were performed in specially prepared microreactors molded from PDMS, designated PDMS (Sylgard™ 184 silicone elastomer; Dow Corning) poured onto silicon wafer molds. The microreactor molds were prepared using 4-in. silicon wafers of Type P, crystal orientation of <1-0-0>, resistivity of 1 to 2 Ω, and thickness of 457–575 μm from Silicon Quest (Santa Clara, CA). After preparation, mixtures of urease enzyme and PDMS (designated PDMS-E) were poured onto the microreactor mold and allowed to cure at ambient conditions.

A negative photoresist, SU-8 (Microchem), was used in the microreactor mold process for preparing the PDSM-E microreactors. When exposed to ultraviolet light, material may be removed via a wet etching process leaving high-definition features in micrometer dimensions. Additionally, a microreactor has been constructed in silicon onto which layer-bylayer self-assembled polyelectrolytes and enzymes are deposited. This system is being used for comparison with the PDMS-E system performance.

Scanning electron microscopy (SEM) images were taken with an AMRAY, 1800 series scanning electron microscope having a resolution of 0.2 μm. All objects in this work, except the silicon wafer microreactor, were first sputtered with a nickel layer a few nanometers thick in order to obtain an SEM image.

Preparation of Biomaterial and Layer-by-Layer Self-Assembly

The combination of PDMS and urease enzyme to form a microreactor from the resulting "bioplastic" material (PDMS-E) has been reported previously *(7)*. When enzyme concentrations were maintained at 2.5% (w/w) or less, the resulting microreactor cured with good structural integrity and high definition (e.g., well-formed microchannels and >90% retention of triangular transverse packing features in the microchannels).

For enzyme attachment to the silicon microreactor tested, a layer-by-layer technique was employed to build a multilayer system of polyions and enzyme. Deposition of multilayers was accomplished by alternating positively and negatively charged layers of polydimethyldiallyl ammonium chloride (PDDA) and polystyrene sulfonate (PSS), respectively, to which was attached urease enzyme. After depositing in succession three layers of PDDA, PSS, and PDDA, three layers of urease enzyme were alternately deposited with three layers of PDDA. The resulting architecture is described as follows:

$$PDDA/PSS/PDDA + (UR/PDDA)_3$$

Batch Studies for Evaluating Enzyme Immobilization and Pdms-E Characteristics

To assess the enzymatic activity of the PDMS-E complex, "nonstructural" PDMS preparations with various enzyme fractions were prepared and cured in glass petri dishes. On curing, these preparations were removed and cut into cubes nominally 3 mm on a side. Equal weights of these cubes (~10 g/reactor) were placed in 250-mL shake flasks. A 150-mL preparation of 0.1 mol/L THAM buffer solution containing 0.1 mol/L of urea was placed in each of three flasks. The pH of the buffer/urea medium was adjusted to 7.5 by the addition of HCl. Shake flasks were placed on a shaker incubator at 25°C and 200 rpm. Sample volumes of 2 mL were removed periodically for ammonia analysis. Additionally, swelling studies were conducted by periodically removing PDMS-E cubes from the urea solution, removing surface water, and weighing to assess the degree of water adsorption by the biopolymer *(8)*.

Microreactor Fabrication

PDMS-E microreactor

The devices employed in this work were fabricated using silicon wafers as either microreactors or molds for the PDMS-E. The silicon wafers were treated by standard photolithographic techniques to form the desired features. Process steps to fabricate micromolds have been presented elsewhere *(8)*. Figure 1 depicts a PDMS-E microreactor after curing on a silicon wafer mold. Figure 2 shows an SEM micrograph of a portion of the microchannel with triangular features fixed within the channel. PDMS-E microreactors were fabricated in 50-, 500-, and 1000-mm lengths to study

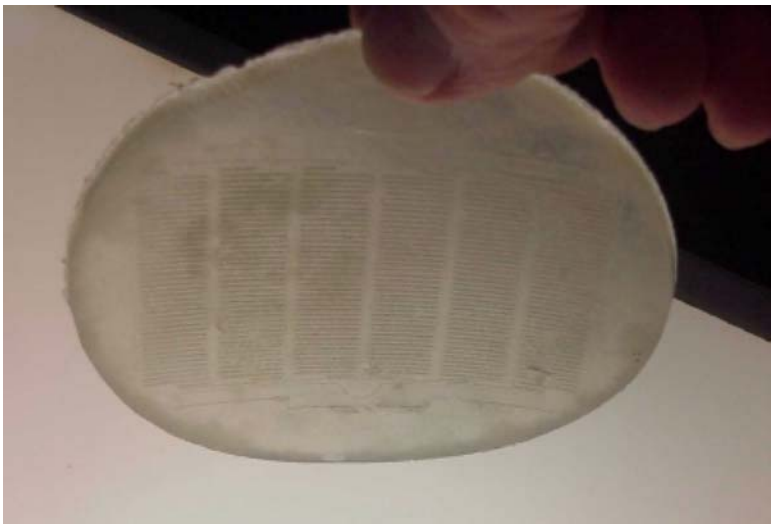

Fig. 1. PDMS-E microreactor containing 1.7 wt% urease.

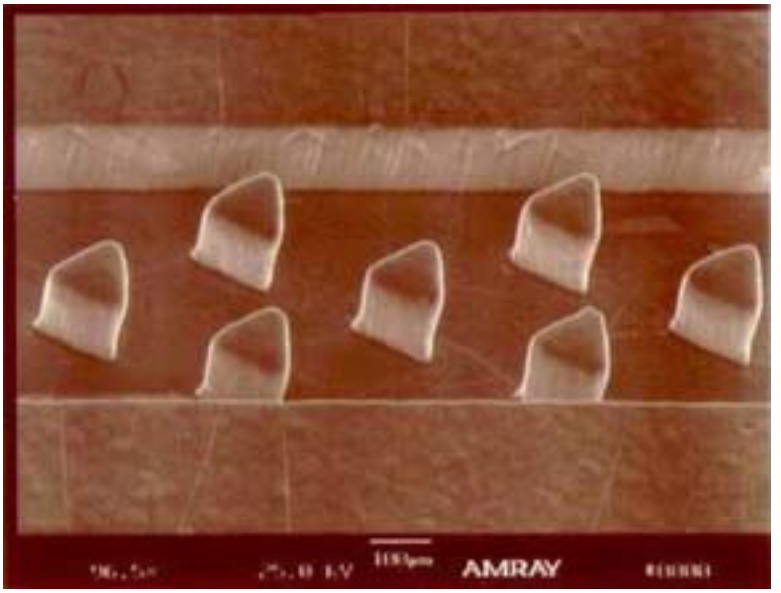

Fig. 2. SEM micrograph of portion of the microchannel with triangular features.

performance characteristics. Table 1 gives the various dimensions and operating conditions under study. Each microreactor enzyme loading was tested at various flow rates. Mean residence times were calculated taking into account the volume occupied by the triangular mixing features.

Table 1
Dimensions and Operating Conditions of PDMS-E Microreactor

Reactor Description	Channel width (μm)/length (mm)	Per channel volume (mm^3)/surface area (mm^2)	Enzyme "loading" g"E"/g PDMS	Urea Feed Solution	
				Flow rate (mL/min)	Mean residence time (min)
1a Six channels (with triangular transverse features)	500/50	2.67/48.3	0.25	0.060	0.27
			0.50	0.006	2.67
			0.75	0.001	16.01
2a Six channels (with triangular transverse features)	500/500	27.7/484.8	0.25	0.060	2.77
			0.25	0.023	7.28
			0.50	0.006	27.65
			0.50	0.001	165.56
3a Six channels (with triangular transverse features)	500/1000	55.4/970	0.25	0.048	6.92
			0.50	0.023	14.56
			0.75	0.006	27.65

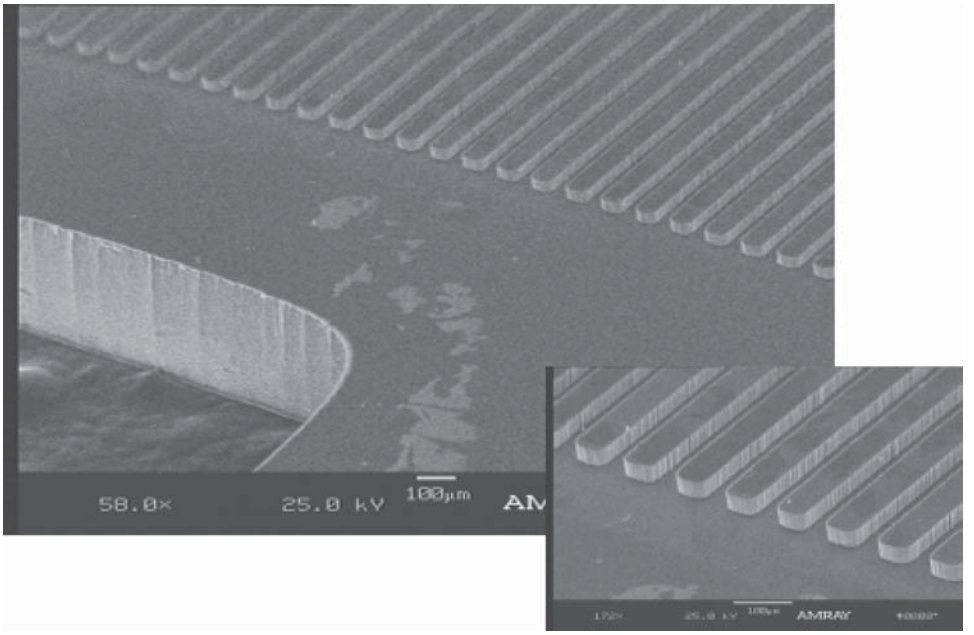

Fig. 3. Silicon wafer microreactor.

Silicon Wafer–Based Microreactor

For comparison of performance, a microreactor was constructed by directly etching microchannels into a silicon wafer using standard lithographic processes. An emulsion mask of the design template was produced using a high-resolution printer and transferred to a chrome mask for alignment and exposure steps. The design was transferred to a <1,0,0> silicon wafer using positive photoresist. The channels were then etched via inductive coupled plasma utilizing the Bosch process that allows microscale perpendicular walls to be fabricated with high aspect ratios (i.e., height/width ratios). Figure 3 shows a portion of the silicon wafer microreactor, and the dimensions for the microreactor are given in Table 2. The experimental system is shown in Fig. 4. Syringe pumps are used to create a precise, slow, and repeatable flow. Direct comparison of this microreactor configuration with the PDMS-E microreactor is the emerging focus of this project for both reactor performance and process economics.

Biochemical Reaction and Microreactor Testing Systems

Urease converts urea to ammonia and carbon dioxide by the following reaction:

$$NH_2CONH_2 + H_2O \text{ g } 2NH_3 + CO_2$$

For the urease enzyme system, a reactant solution of 0.1 mol/L of urea was fed to the microreactors by Cole Parmer Series 74900 Syringe pumps.

Table 2
Characteristics of Silicon Wafer Microreactor

Length	2.7 cm
Width	0.5 cm
Channel width	50.0 µm
Channel depth	100.0 µm
Number of channels	98

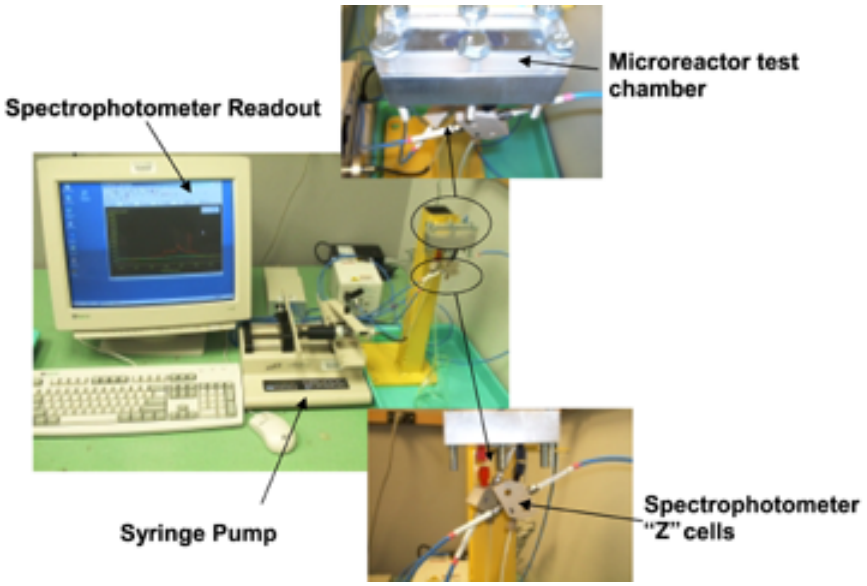

Fig. 4. Experimental setup for testing silicon wafer microreactors.

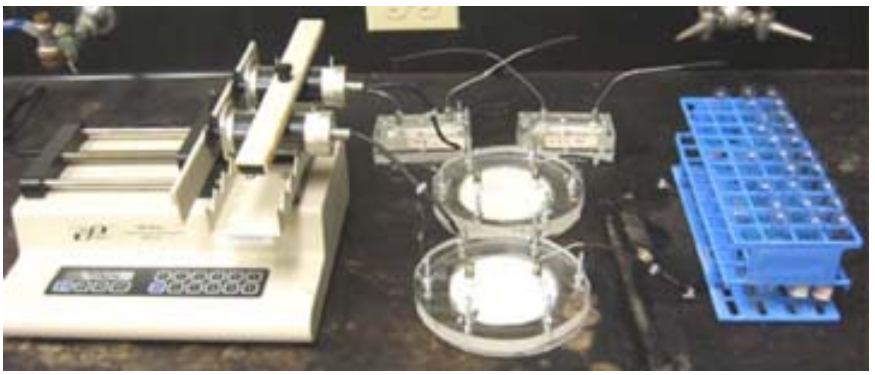

Fig. 5. Experimental setup for testing PDMS-E microreactors.

The experimental setup for the PDMS-E microreactor system is shown in Fig. 5. Reactor effluent was analyzed by a Hewlett Packard 1100 HPLC (UV-Vis detector) and an Ocean Optics SD 2000 UV-Vis Spectrometer with fiberoptic flow analysis "Z" cells (FIA Lab).

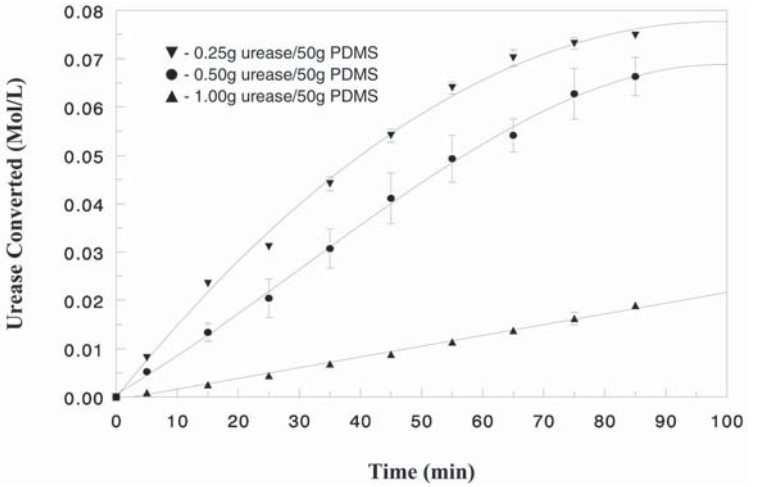

Fig. 6. Urea conversions for various enzyme loadings in PDMS in batch reactors.

Results and Discussion

Batch Studies

A series of batch studies was run to test the biomaterial made from urease entrapped in PDMS. Quantities of urease (0.25, 0.50, and 1.00 g, respectively) were each mixed with separate 50-g quantities of PDMS elastomer. Once cured at ambient conditions (25°C, atmospheric pressure), the resulting bioplastic was cut into sections (nominal 3-mm cubes) and added in 10-g quantities to separate 250-mL flasks containing 0.01 *M* urea solution (in THAM buffer at pH ~7.4). Urea conversion and pH were monitored with time. Figure 6 shows initial rates of urea conversion as a function of time. For each data set, flasks were run in triplicate and results were averaged. Error bars are shown for 1 SD from the mean. For the higher enzyme concentration, the pH approached a value of 9.0 in approx 55 min, at which level urease enzyme activity has been shown to be inhibited *(8–9)*. Here, as our batch systems approached this pH value (9.0), a noticeable decrease in reaction rate was observed. Thus, the decline in activity is most likely attributable to pH effects. The increase in the rate of urea conversion was observed to be significant for increased enzyme loadings. The initial reaction rates were calculated to be 0.2, 0.8, and 0.9 mmol/(L·min) for the 0.25-, 0.50-, and 1.00-g enzyme loading, respectively. These rates are in the range of those reported for various urease immobilization techniques *(10–12)*.

Continuous-Flow Studies

The PDMS-E described in the batch studies was used to mold reactors. These microreactors were fed the same 0.1 *M* urea solution as used in batch experiments. Reactors were operated for approx 1 h before acquiring operational data to reduce the effects of any loosely bound enzymes that may wash out from the surfaces of the microchannel walls.

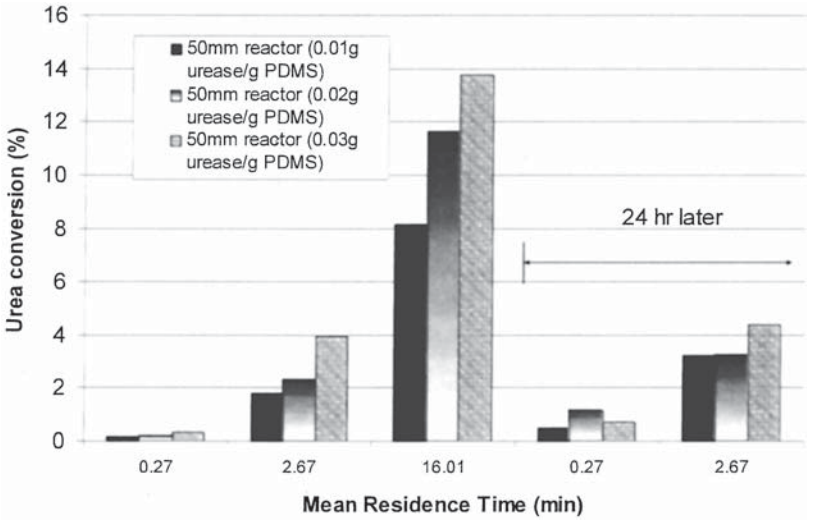

Fig. 7. Effect of enzyme loading on conversion in 50-mm PDMS-E continuous-flow reactors.

The shallow depth of the channels (125 μm in the PDMS-E microreactors and 100 μm in the silicon wafer microreactor) provides for very short reactant diffusion lengths. This is one of the great advantages of microscale reactors. Small cross-channel dimensions also induce laminar flow. All experimental flows in this study had Reynolds numbers below 1.0.

Residence Time and Enzyme Loading

Figures 7 and 8 show conversions in PDMS-E microreactors having 50- and 500-mm channel lengths. In Fig. 7, microreactors with a 50-mm channel length and three different enzyme loadings (0.01, 0.02, and 0.03 g of urease/g of PDMS, respectively) were operated with three different flow rates each (shown as residence time on the *x*-axis) during a 48-h period. In the first 24 h, the microreactor systems showed an increase in urease conversion both with higher residence times and with higher enzyme loadings, supporting the idea of increased enzyme availability in the cured polymeric microstructure. However, during a second 24-h period a significant decline in enzyme activity was noted. While inconclusive, the relative comparison among the three enzyme loadings during the second 24-h period points to a combination of enzyme inactivation and possible detachment of the additional enzyme initially incorporated into the PDMS polymer matrix accompanying the observed decline in enzyme activity. Additional testing is under way to ascertain the potential for enzyme detachment and residual activity in the microreactor effluent. Figure 8 shows a comparison of 50- and 500-mm channel length microreactors for an enzyme loading of 0.01 g of urease/g of PDMS elastomer. To highlight

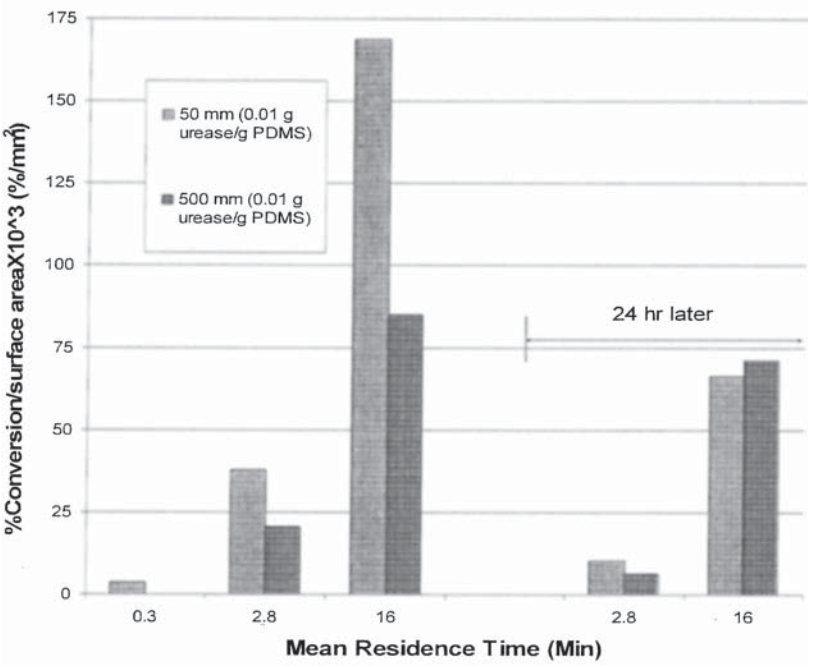

Fig. 8. Effect of residence time on conversion in PDMS-E continuous-flow reactors.

reactor efficiency, conversion per unit of reactor surface area (% conversion/mm2) is plotted vs mean residence time. The relative efficiency of both the 50- and 500-mm microchannel reactors is comparable. For a residence time of 16 min, there is an apparently significant increase in the 50-mm microreactor over the 500-mm microreactor. However, at this residence time, the 500-mm microreactor achieved nearly 100% urea conversion, pointing to the possibility that it may have been "underutilized," thereby exhibiting the comparative inefficiency. As in the previous experiment, a decline in reactor performance was observed during the second 24-h period.

Figure 9 illustrates the operation of 1000-mm microchannel length reactors at enzyme loadings of 0.01 and 0.03 g of urease/g of PDMS and at various volumetric flow rates. Flow rates of 0.023 and 0.073 mL/min correspond to mean residence times of 14.55 and 4.55 min, respectively. Data sets are averages for three runs at each condition. Error bars depict 1 SD from the mean. In all cases, a significant decline in reactor activity was observed after initial startup. However, in the case of the microreactor with a loading of 0.03 g of urease/g of PDMS and a flow rate of 0.073 mL/min, virtually 100% conversion was maintained for 6 h with a gradual decline and leveling off at approx 55%. Generally, activity seems to level off at about 50% after about 24 h. This behavior is similar to the decline in enzyme reactivity for other immobilized enzyme systems (*see* e.g. ref *13*).

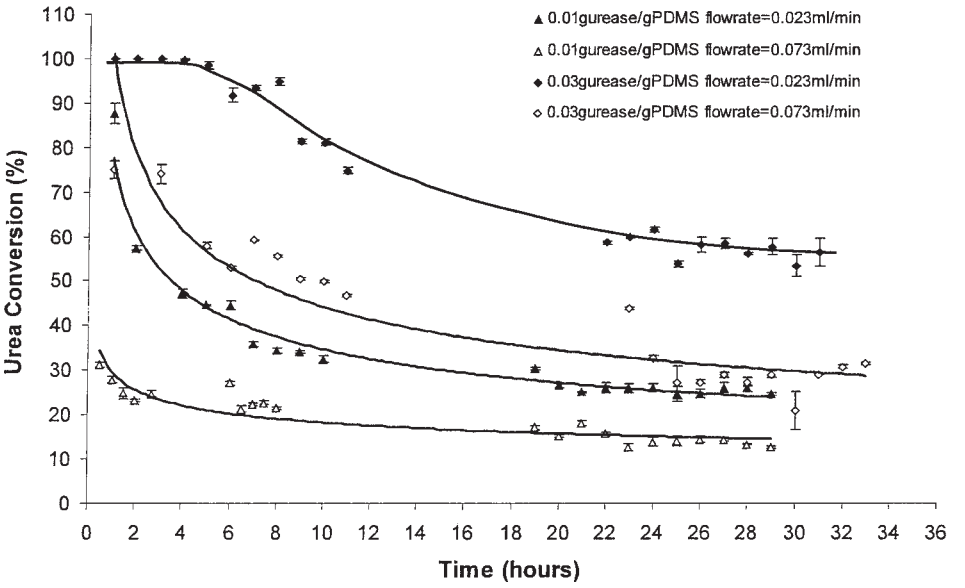

Fig. 9. Effect of time on conversions for 1000-mm PDMS-E continuous-flow reactor with various enzyme loadings.

Silicon Microreactor

While per-unit cost for a silicon wafer microreactor is higher than the PDMS-E microreactor system, silicon-based devices may have significant advantages. Since PDMS is extremely inert, it would be very difficult to attach enzymes to its surface. The layer-by-layer selfassembly techniques to apply enzymes to silicon surfaces allow for a higher population of enzymes on the surface than entrapment can provide. Assuming a cubic array of perfectly mixed enzymes in a PDMS matrix, there are approx 10^{10} enzymes/cm^2 on the surface. For enzymes of about 10 nm in diameter, complete surface coverage is 10^{12} enzymes/cm^2. Enzymes immobilized by self-assembly may also display a greater stability than those entrapped in PDMS. For comparison to the PDMS-E system, an additional study is under way to examine the performance of a silicon wafer–based microreactor to examine both reaction efficiency and longevity of performance. In this system, a technique of layer-by-layer self-assembly *(14–17)* is used for attaching enzymes to the microchannel surface. Preliminary data show high urea conversions after a significant "shelf life." For example, silicon microreactors >1 mo old (stored at 4°C) have exhibited significant enzyme activity. Future reports will describe this work in detail.

Conclusion

Continuous-flow microreactors were successfully fabricated from PDMS and entrapped urease. Conversions increased almost proportion-

ally with an increase in residence time. Conversions increased significantly with enzyme loading. Over a 30-h period of continuous operation, conversions decreased by about half and then leveled off.

Continuous-flow microreactors were successfully fabricated by etching channels in silicon and immobilizing urease onto channel surfaces by a layer-by-layer self-assembly technique. Preliminary results show urea conversion. The potential advantages of this surface-coating technique in microreactors warrant continued investigation.

Acknowledgments

We acknowledge Dr. Yuri Lvov at Louisiana Tech's Institute for Micromanufacturing for assistance in applying layer-by-layer self-assembly techniques. We also wish to thank the Department of Defense (DAAH04-96-1-0200) and the Louisiana Board of Regents (LEQSF [1999-02]-RD-A-24) for their support of this work.

References

1. Srinivasan, R., Hsing, I.-M., Berger, P. E., Jensen, K. F., Firebaugh, S. L., Schmidt, M. A., Harold, M. P., Lerou, J. J., and Ryley, J. (1997), *AIChE J.* **43(11),** 3059–3069.
2. Wegeng, R., Call, C., and Drost, K. (1996), *Chemical System Miniaturization*, PNNL-SA-27317, Pacific Northwest National Laboratory, Richland, WA.
3. Schuth, F. (1995), *Crystallographically Defined Pore Systems: Reaction Vessels with Molecular Dimensions*, Microsystem Technology for Chemical and Biological Microreactors, Max Planc Institute, Mainz, Germany.
4. Zheng, A.-P., Jones, F., Fang, J., and Cui, T. (2000), in *Proceedings of the 4th International Conference on Microreaction Technology (IMRET IV)*, Atlanta, GA, pp. 284–292.
5. Palmer, J., Ramprasad, S., and Forrest, S. (2003), *TexMEMS V*.
6. Palmer, J., Ramprasad, S., and Forrest, S. (2003), in *Proceedings for the AIChE Spring 2003 National Conference*, New Orleans, LA.
7. Laidler, K. J. and Hoare, J. P. J. (1950), *Amer. Chem. Soc.* **72,** 2487–2494.
8. Jones, F., Lu, Z., and Elmore, B. B. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 627–640.
9. Ramachandran, K. B. and Perlmutter, D. D. (1976), *Biotechnol. Bioeng.* **18,** 685–699.
10. Dumitriu, S., Popa, M., Artenie, V., and Dan, F. (1989), *Biotechnol. Bioengin.* **34,** 283–290.
11. Moynihan, H. J., Lee, C. K., Clark, W., and Wang, N. H. L. (1989), *Biotechnol. Bioengin.* **34,** 951–963.
12. Schussel, L. J. and Atwater, J. E. (1995), *Chemosphere* **30(5),** 985–994.
13. Subramanian, A., Kennel, S. J., Oden, P. I., Jacobson, K. B., Woodward, J., and Doktycz, M. J. (1999), *Enzyme Microb. Technol.* **24,** 26–34.
14. Hua, F., Shi, J., Lvov, Y., and Cui, T. (2002), *Nano Lett.* **2,** 1219–1222.
15. Chang-Yen, D., Lvov, Y., McShane, M., and Gale, B. (2002), *Sens. Actuators B* **87,** 336–345.
16. Fang, M., Grant, P., McShane, M., Sukhorukov, G., Golub, V., and Lvov, Y. (2002), *Langmuir* **18,** 6338–6344.
17. Hua, F., Lvov, Y., and Cui, T. (2002), *Nanosci. Nanotechnol.* **2,** 257–361.